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Transmitter-Specific Subsets of Sensory Elements in the Prosobranch Osphradium

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Abstract. The osphradium is a putative chemosensory organ of aquatic molluscs. Previously, we identified two distinct types of primary sensory neurons in the osphradial ganglion of freshwater pulmonates, one immunoreactive to leucine-enkephalin (LEnk-ir) and another to FMRFamide (FMRFa-ir). In addition, NADPH diaphorase (NADPHd)-positive elements apparently producing nitric oxide (NO) were demonstrated in the organ. In the present study, prosobranch molluscs, which have retained the osphradial sensory neurons within the epithelium, were studied. Both types of peptidergic neurons, as well as NADPHd-positive cells, were found within the epithelium or in a basiepithelial position in the relatively simple osphradium of the mesogastropod *Littorina littorea* and in the complex, bipectinate osphradium of the neogastropod *Buccinum undatum*. Similar evidence was also obtained for another mesogastropod, *Ampullarius* sp. Transmitter-specific sensory cell types like those discovered in the osphradium are also present as single neuroepithelial cells in other organs of the mantle complex in prosobranchs and in the pelecypod *Anodonta cygnea*. We suggest that evolutionarily conservative, transmitter-specific types of epithelial and neuroepithelial sensory cells predated the osphradium, which developed as the site of their concentration while retaining characteristic subsets of sensory neurons.

Introduction

The osphradium is a molluscan sensory organ characteristic mainly of aquatic forms. Evidence suggesting its chemosensory function has been considered and summarized (Sokolov *et al.*, 1980; Croll, 1983; Haszprunar, 1985; Emery, 1992). The position and common structural features shared by osphradia of various molluscs indicate a homology of the organ across the phylum (Haszprunar 1985, 1987).

According to our previous findings in a freshwater pulmonate, *Lymnaea stagnalis*, two distinct types of osphradial sensory neurons can be distinguished immunocytochemically, one labeled with an antiserum against FMRFamide (FMRFa-ir), and another immunoreactive to an antiserum against leucine-enkephalin (LEnk-ir). Cell bodies of these primary sensory neurons are situated within the osphradial ganglion, and their distal processes penetrate the sensory epithelium. Other, nonsensory neurons within the ganglion contain neuroactive substances that were demonstrated immunocytochemically, with antisera against serotonin and methionine-enkephalin (MEnk-ir), and histochemically, with a procedure for NADPH diaphorase (NADPHd) (Elofsson *et al.*, 1993; Nezlin *et al.*, 1994).

Transmitter specificity is claimed to be a conservative feature of homologous nerve cells (Sakharov, 1970). We test this idea in a comparative study of immunocytochemically identified sensory cells: we ask whether nonpulmonate osphradia share common transmitter-specific neuron phenotypes with those of pulmonates. In addressing this question, we chose prosobranch molluscs for two reasons. First, unlike evolutionarily advanced pulmonates, most of the prosobranchs retain their osphradial sensory neurons in the supposed initial position—within the sensory epithelium of the organ (Haszprunar, 1985). Second, prosobranch osphradia demonstrate a wide range of complexity, ranging from a hardly noticeable structure in *Littorina littorea*, to a large, gill-like organ—the so-called

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Abbreviations: FMRFa-ir, FMRFamide-like immunoreactive; LEnk-ir, leucine-enkephalin-like immunoreactive; MEnk-ir, methionine-enkephalin-like immunoreactive; NADPHd, NADPH diaphorase; NO, nitric oxide.

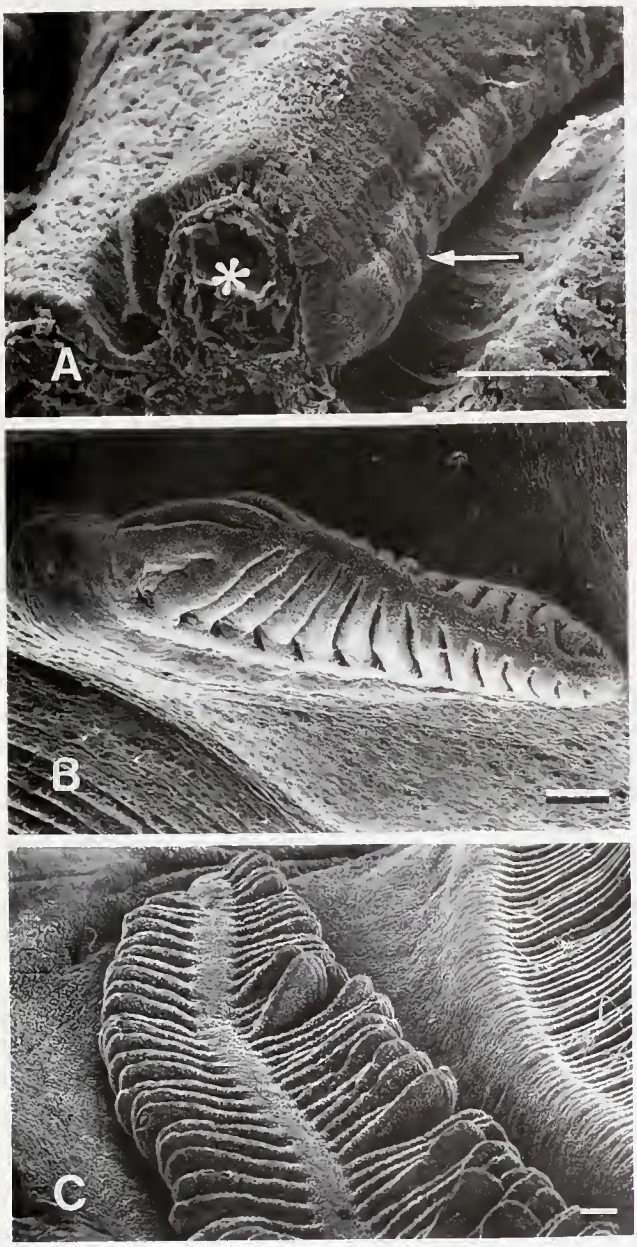

Figure 1. Scanning electron micrographs of the osphradium in the prosobranchs studied. A. *Littorina littorea.* A transverse section has been made through the osphradium. Asterisk labels the ganglion, and the arrow indicates the lateral ciliated zone of the ridge. B. *Ampullarius* sp. C. *Buccinum undatum.* Scale bars: A = 100 μm; B, C = 200 μm.

bipectinate osphradium—in *Buccinum undatum.* Our comparative study also included the morphologically peculiar organ of an ampullariid snail (Haszprunar, 1985) and the mantle epithelia surrounding the prosobranch osphradium as well as that of a pelecypod, the freshwater mussel *Anodonta cygnea,* which has a poorly developed osphradium.

Materials and Methods

Specimens of the periwinkle *Littorina littorea* (Prosobranchia, Mesogastropoda, Littorinoidea), 2–2.5 cm long, and the whelk *Buccinum undatum* (Prosobranchia, Neogastropoda, Buccinoidea), 7–9 cm long, were collected in the wild at the Kristineberg Marine Biological Station on the west coast of Sweden, and maintained in aquaria for about two weeks at about 10°C. Specimens of the freshwater snail *Ampullarius* sp. (Prosobranchia, Mesogastropoda, Viviparoidea) and the pelecypod *Anodonta cygnea* were from an aquarial culture. To dissect osphradia and surrounding tissues, animals were anesthetized in an isotonic solution of $MgCl_2$ for 20 min and then extracted from their shells.

Scanning electron microscopy

The osphradia were fixed in 2.5% glutaraldehyde (TAAB) in 0.05 *M* Na-cacodylate buffer, pH 7.3 (23 mg/ml NaCl were added for the marine species), for 2 h, and washed in the same buffer for 2 h. To remove mucus from the surface, the specimens were treated for 6 h with leech hyaluronidase (Sigma), 50–100 units/ml, in the same buffer. All steps were at room temperature. Finally, the specimens were dehydrated in an ethanol series, dryed in Balsers Union Critical Point Dryer, fixed to stubs, and coated with about 14 nm of gold in a Bio-Rad Polaron SEM Coating System. Observations and micrographs were made with a JEOL JSM-T330 electron microscope.

Immunolabeling

The dissected osphradia were fixed for 2 h at 4°C in 4% paraformaldehyde dissolved in 0.2 *M* (0.1 *M* for *Ampullarius*) Na-phosphate buffer solution (PBS), pH 7.4, washed overnight at 4°C in PBS, immersed for 1 h at 4°C in 20% sucrose in the same buffer, embedded in Tissue Tek (Miles), and frozen in liquid nitrogen. Serial sections were cut at a thickness of 20 μm with a Reichert-Jung Frigocut E 2800 cryostat and placed on chrome-alum/gelatin-coated glass slides. The slides were air-dried for 15 min, washed in PBS containing 0.25% Triton X-100 (TX), and treated at room temperature as follows:

1. Incubate for 30 min with 10% normal swine serum, diluted in PBS-TX containing 0.2% Bovine Serum Albumine (BSA, Sigma).
2. Wash in PBS-TX (3 × 20 min).
3. Incubate with primary antiserum, 1:1000, in PBS-TX-BSA (6 h).
4. Wash in PBS-TX (3 × 20 min).
5. Incubate with swine anti-rabbit immunoglobulins, 1:50, in PBS-TX-BSA (2 h).

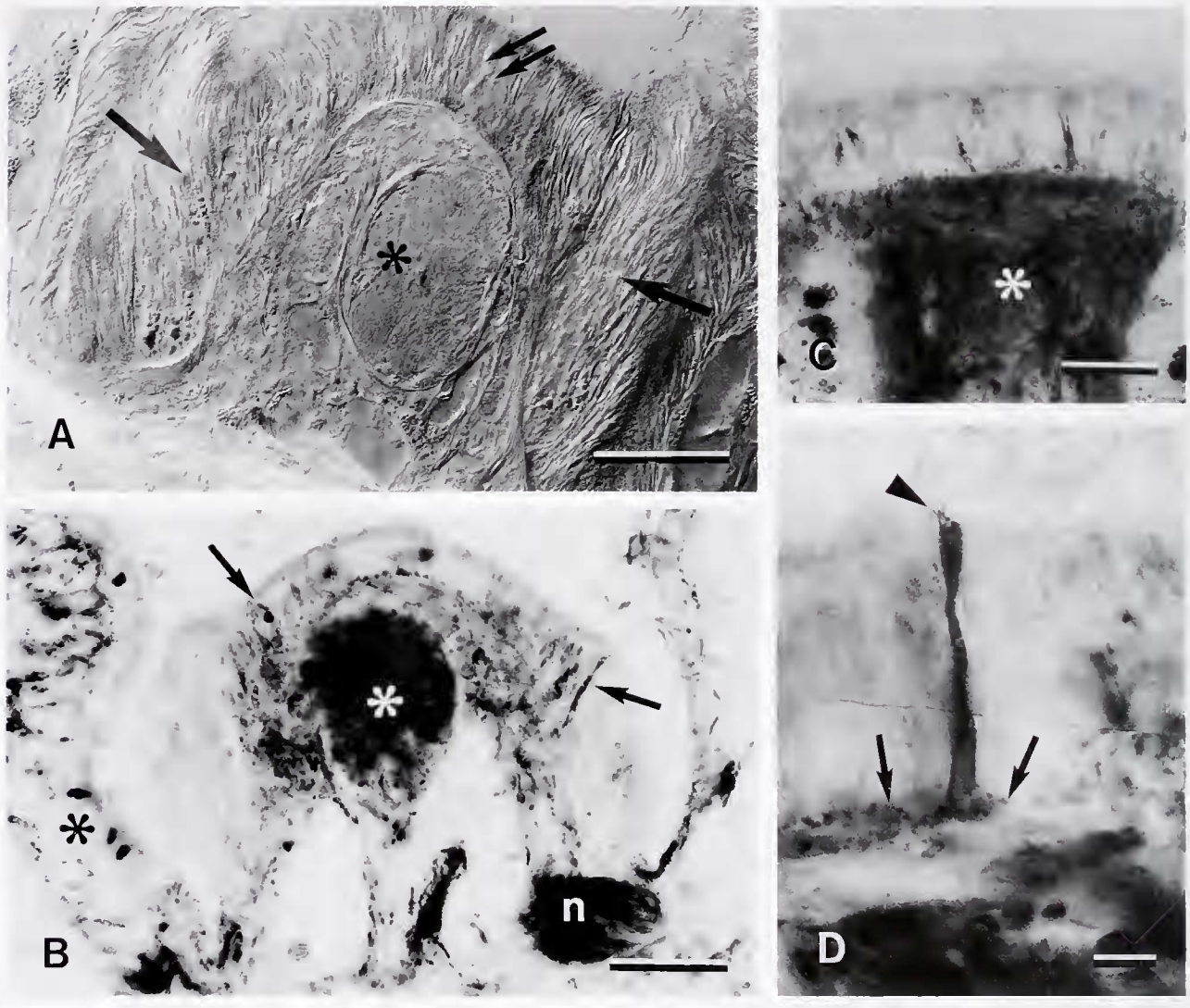

Figure 2. Transverse sections through the osphradium of *Littorina littorea*. (A) General view of an unstained specimen, interference contrast. Asterisk, the osphradial ganglion; arrows, lateral ciliated epithelium facing the groove; double arrow, the central epithelial zone. (B–D) FMRFa-ir elements, peroxidase labeling. (B) Section similar to A. White asterisk, the osphradial ganglion; black asterisk, non-specifically labeled cells in the epithelium; arrows, immunopositive epithelial cells; n, the osphradial nerve. (C) Central portion of the osphradium with immunopositive epithelial cells and intensive labeling in the ganglion (white asterisk). (D) Neuroepithelial cell in the central epithelial zone with two basal processes (arrows) and apical protrusions (arrowhead). Scale bars: A–C = 50 μm; D = 10 μm.

6. Wash in [illegible] (3 × 20 min).
7. Incubate with [illegible]dase-anti-peroxidase complex, 1:100, in PBS-TX-BSA (2 h).
8. Wash in PBS-TX (3 × 20 min).
9. Wash in 0.1 *M* Tris-HCl buffer solution (TBS), pH 7.4 (2 × 15 min).
10. Incubate in 0.05% diaminobenzidine (DAB) in TBS (20 min).
11. Incubate in DAB with 0.01% H_2O_2 in TBS (20 min).
12. Wash in TBS (3 × 20 min).
13. Dehydrate in an ethanol series, and embed in Permount (Fisher Scientific).

After stage 3, some slides were treated as follows:

4. Wash in PBS (3 × 20 min).
5. Incubate with fluorescein-(FITC)-labeled swine anti-rabbit immunoglobulins, 1:50, in PBS-BSA (2 h).
6. Wash in PBS (3 × 20 min).
7. Embed in PBS-glycerol (1:1).

The slides were examined with a Leitz-Aristoplan Universal microscope. Epifluorescence equipment (excitation maximum 490 nm, emission barrier >515 nm) was used for FITC-labeled specimens. Polyclonal antisera raised against FMRFamide-, leucine-enkephalin-, and methionine-enkephalin-conjugates were purchased from Incstar (Stillwater, Minnesota). Controls included the omission of primary antibody, or its replacement with normal rabbit serum.

NADPH-diaphorase histochemistry

The osphradia were fixed in paraformaldehyde in PBS and sectioned as above. The slides were washed (2 × 15 min) in 0.05 *M* TBS (0.5 *M* for marine species), pH 8.0, at room temperature and treated according to a standard histochemical procedure. They were incubated in the darkness for 1 h, at room temperature or at 37°C, in TBS containing 0.8 mg/ml β-NADPH (Sigma), 0.4 mg/ml Nitro Blue Tetrazolium (BDH), and 0.2% TX-100. In the control solutions, β-NADPH was replaced with α-NADPH, Sigma, in the same concentration (Hope and Vincent, 1989). Then the slides were rinsed in TBS followed by distilled water, dehydrated through an ethanol series, and embedded in Entellan (Merck).

Results

In all three prosobranch species examined, the osphradium is situated in the mantle roof, at the base of the siphon, which means that it is exposed to the inhalant current of external water. Although the outer morphology varies considerably (Fig. 1A–C), the internal structures correspond to a great degree. The main constituents of the osphradium are an elongated axial cord of the nervous tissue, the osphradial ganglion, and a specialized epithelium containing several types of neuroepithelial cells (Crisp, 1973; Haszprunar, 1985). The organ also contains connective tissue, muscle fibers, and hemocoel. The ganglion is connected with the CNS via one or more branches of the supraintestinal nerve. In the epithelium, sensory (central), ciliated, and glandular zones can be recognized (Hyman, 1967).

The control preparations could be used to identify the background and unspecific staining of *Littorina* and *Buccinum* tissues by both immunocytochemical and histochemical methods. *Ampullaris*, however, is a special case (see below).

Littorina littorea

The osphradium is a relatively simple structure running alongside the left gill. Its form is that of a long ridge rising from the bottom of a groove (Fig. 1A, 2A, B). The central zone of the epithelium is at the top of the ridge, above the sausage-like osphradial ganglion. On each side of the ridge is a band of thickened epithelium identified as the ciliated zone (Crisp, 1973; Haszprunar, 1985) (Fig. 2A). Glandular zones have not been clearly indicated by earlier authors.

FMRFa-ir elements. Labeled cells were consistently seen both in the osphradial epithelium and in the ganglion (Fig. 2B). In the epithelium, they are represented by neuroepithelial cells (Fig. 2C) giving rise to a proximal neurite (Fig. 2D). The cells appear differently in various parts of the epithelium. In the central zone, they are oblong and terminate with a few short protrusions resembling reduced cilia (Fig. 2C, D). In the lateral ciliary zone, they are extremely thin and elongated (Fig. 2B, arrow). Proximal neurites extending from epithelial FMRFa-ir cells of the central zone could be traced into the basal plexus, which is connected to the osphradial ganglion. Within the ganglion, these afferent projections and processes of intrinsic FMRFa-ir neurons form a dense FMRFa-ir neuropile.

LEnk-ir elements. Neuroepithelial cells of this type show two distinct sites of localization in the osphradial epithelium. One is a narrow band of cells on top of the ridge. Within the epithelium, these multipolar LEnk-ir cells send fine processes in different directions (Fig. 3A). The other site is a band along the lateral sides of the ridge (Fig. 3B). In this case, the cell bodies are situated deep in the epithelium close to the base of the ridge and send long processes to the surface. Groups of similar basiepithelial LEnk-ir cells are consistently present in the wall of the groove and, more peripherally, in adjacent portions of the mantle wall (Fig. 3C). Fibers from LEnk-ir neuroepithelial cells concentrate mostly around and in the outer zone of the osphradial ganglion. The intrinsic neuronal population of the ganglion includes a small number of scattered LEnk-ir neurons.

MEnk-ir elements. These are absent from the osphradial epithelium. They appear as a fiber meshwork and, occasionally, as cell bodies under the epithelium and in the osphradial ganglion (Fig. 3D). In the mantle wall outside the osphradium, MEnk-ir elements are sometimes well developed, particularly in the connective tissue.

NADPHd-positive elements. The mantle epithelium on each side of, and adjacent to, the osphradium is thicker than that of the surrounding mantle wall and more closely resembles the osphradial epithelium. In this area of the mantle, a band of scattered cells stained with β-NADPH (Fig. 4A) lies close to the osphradium and just outside the groove. The cells are elongated and sometimes possess a thin distal process, but they are apparently devoid of a proximal, basal neurite. NADPHd-positive epithelial cells were otherwise absent from the mantle except for a narrow region of the so-called "mantle ridge," the outer edge of

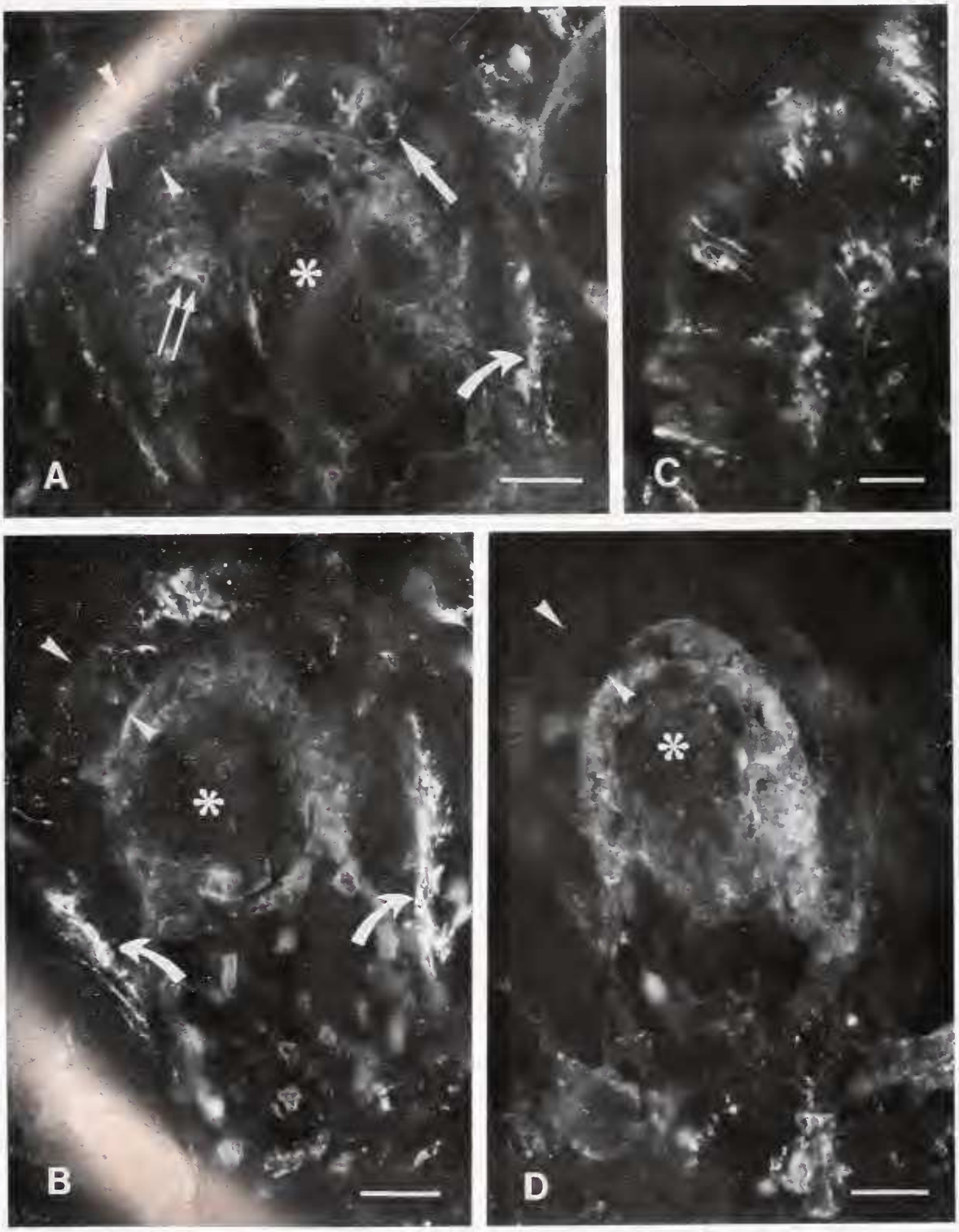

Figure 3. Enkephalin immunoreactivity in transverse sections of the osphradium of *Littorina littorea*. FITC-labeling. (A) LEnk-ir elements. Asterisk, the osphradial ganglion; arrowheads, the borders of the central epithelium; arrows, immunopositive neuroepithelial cells with branched processes; double arrow,

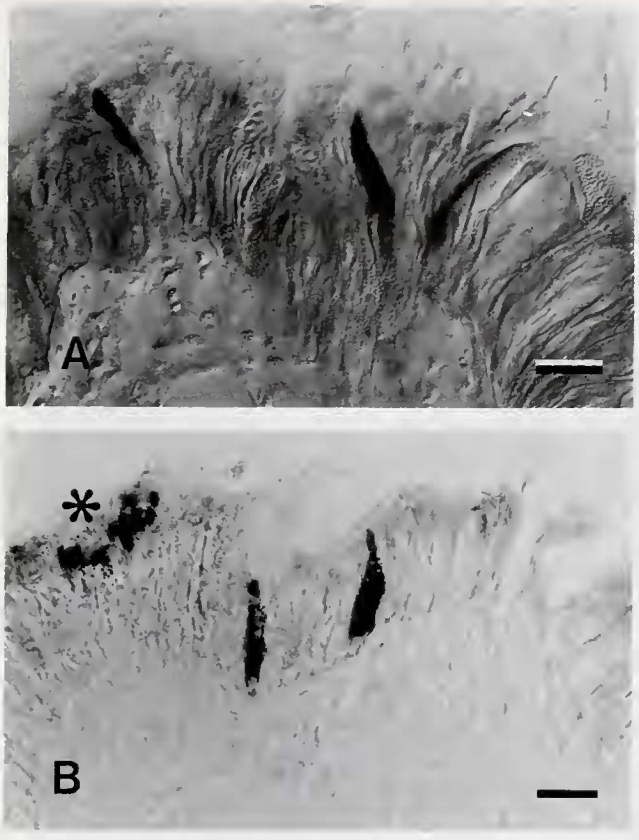

Figure 4. NADPHd-positive cells in the epithelium of the mantle wall adjacent to the osphradium (A) and of the mantle ridge (B) of *Littorina littorea*. In A, the osphradial groove starts just to the right of stained cells. Asterisk in B indicates pigment granules in the epithelium. Scale bars = 10 μm.

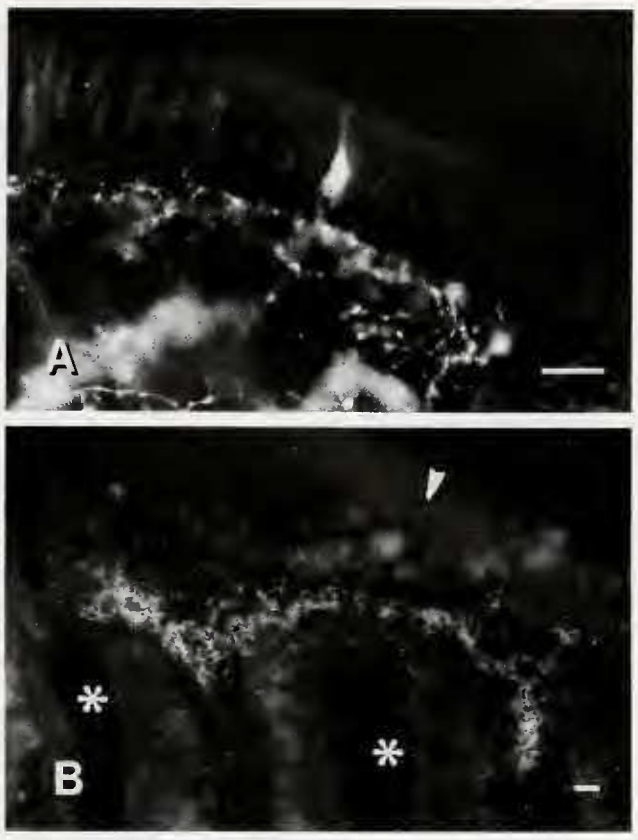

Figure 5. Immunoreactivity in a transverse section of the osphradium of *Ampullarius* sp., FITC-labeling. (A) FMRFa-ir cell body in the epithelium of the central zone with a dense plexus beneath. (B) LEnk-ir fibers in a horizontal section of the outer wall of the osphradial pockets (asterisks). Arrowhead, the outer boarder of the epithelium. Scale bars = 20 μm.

the mantle (Fig. 4B). In control preparations no staining was observed.

Ampullarius sp.

The osphradium of this mesogastropod is more complicated than that of *Littorina*. It is essentially a large oblong fold elevated over the mantle wall. A long, sausage-shaped ganglion is centrally located in the osphradium, and a broad lamella of the nervous tissue runs dorsally along the ganglion. Narrow pockets, perpendicular to the ganglion and aligned with each other on both sides (Fig. 1B), form a lamellar appearance in horizontal sections through the organ.

FMRFa-ir elements are abundant and have their cell bodies in the epithelium. Most of the cells are situated in the epithelium surrounding the ventral portion of the pocket. A smaller fraction is present in the central zone of the osphradial epithelium situated on the top of the organ (Fig. 5A).

LEnk-ir elements appear as very fine fibers in the epithelium of the outer ventral wall of the pockets (Fig. 5B). The cell bodies have a basiepithelial position and are also

Figure 3. (*Continued*) cell body in the ganglion; curved arrow, lateral band of immunopositive cells in the ciliated epithelium. (B) LEnk-ir elements. Curved arrows demonstrate two symmetrical lateral bands of immunopositive cells. White asterisk, the osphradial ganglion; arrowheads, the borders of the central epithelium. (C) LEnk-ir cells in the epithelium of the mantle wall adjacent to the osphradium. (D) MEnk-ir elements. White asterisk, the osphradial ganglion; arrowheads, the borders of central epithelium. Scale bars: A, B, D = 50 μm; C = 10 μm.

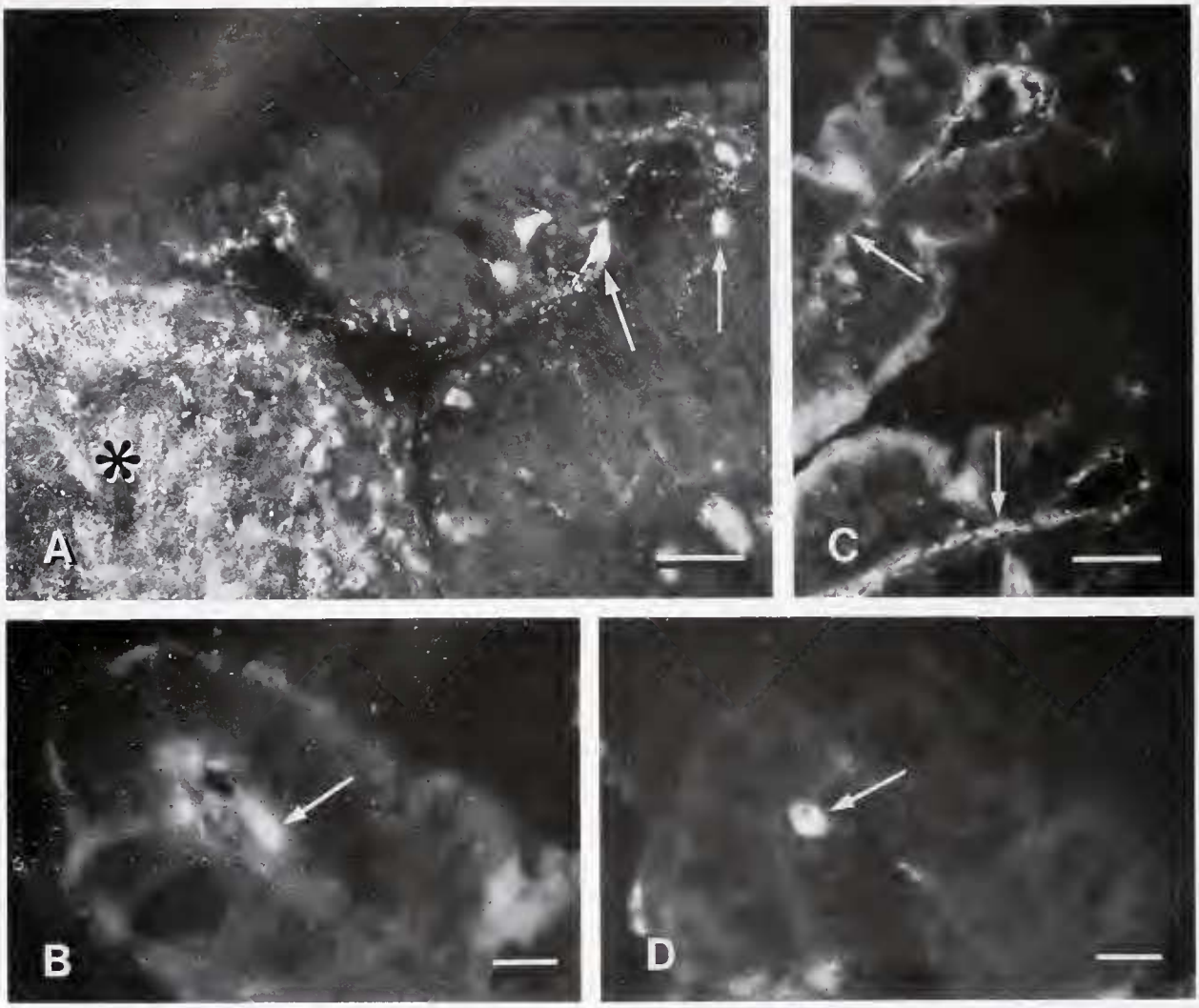

Figure 6. Immunoreactivity in the osphradium of *Buccinum undatum*. FITC-labeling. (A) FMRFa-ir elements in a transverse section of the osphradial ganglion (asterisk) and the medial part of the lamella; arrows, basiepithelial cells in the lamella. (B, C) LEnk-ir elements in a transverse section of the peripheral part of osphradial lamellae. Arrows: in B indicates the cell body; in C, immunopositive processes. (D) MEnk-ir elements in a transverse section of the peripheral part of the osphradial lamellae. Arrow shows a cell body. Scale bars: A, C = 50 μm; B, D = 20 μm.

placed a long distance away, probably close to the ventral surface of the ganglion.

MEnk-ir elements represented only by nerve fibers concentrate in small islets in the connective tissue of the organ.

NADPHd-positive elements are shown as an intensive staining of the outer portion of the osphradial epithelium, including the layer occupied by cilia. In addition, solitary nerve cell bodies and fibers in the ganglion were stained. With α-NADPH as a substrate, the entire osphradial epithelium was diffusely stained.

Buccinum undatum

Among Prosobranchia, neogastropods have the most sophisticated osphradium. Due to pectination, it resembles a gill. In the bipectinate osphradium of *B. undatum*, narrow folds called leaflets, or lamellae, extend laterally from a central core in a very regular fashion (Fig. 1C). Each lamella receives a branching nerve from the central, sausage-like ganglion. The whole structure is raised well above the epithelium of the mantle.

Figure 1C clearly shows the osphradial lamellae; they are narrow where they join the ganglion-containing central

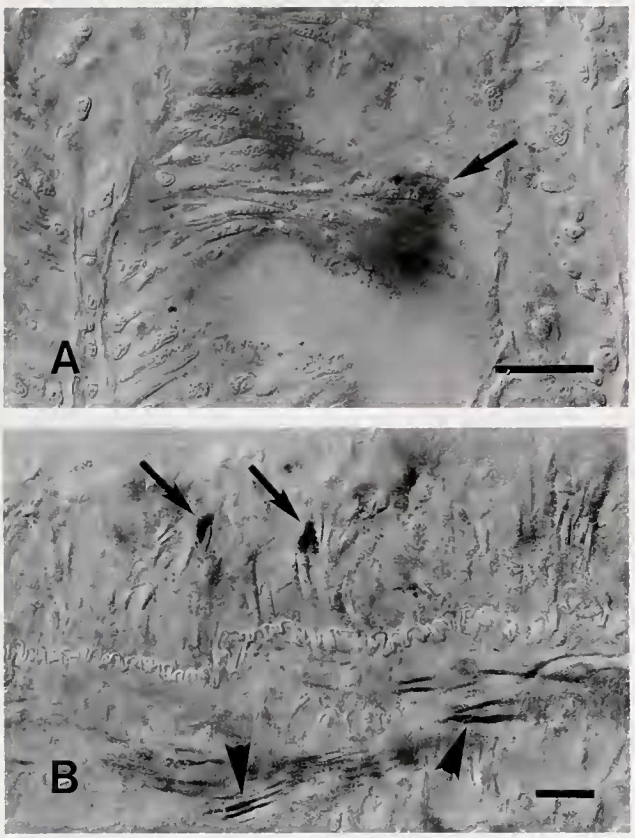

Figure 7. NADPHd-positive cells in the ciliated zone of the lamella (A) and in the mantle wall adjacent to the osphradium (B) of *Buccinum undatum*. Arrows indicate specific staining in apical portions of the epithelial cells; arrowheads, staining in muscle fibers. Scale bars = 20 μm.

core, and wide at the periphery. The glandular zone of the osphradial epithelium runs along the curved outer edge and is tube-like on a cross section (Fig. 6C). This peripheral tube is separated from the main portion of the lamella with a constriction. The ciliary zone is found adjacent to this constriction. The main portion of the lamella is described as a sensory type epithelium, the only type on the central core of the osphradium (Hyman, 1967; Crisp, 1973; Haszprunar, 1985).

FMRFa-ir elements are consistently found in the medio-apical portion of the sensory epithelium of the lamella. They are mostly represented by groups of relatively large, oval or round cells that have a fine distal process facing the mantle cavity and a basal neurite branching in the lamella. Cell bodies occupy a basiepithelial position in the lamella (Fig. 6A). The fibers do not enter the nerve that arises from the ganglion, but approach the ganglion separately. A second site of FMRFa-ir neuroepithelial cells (not represented in Fig. 6A), is found in the central zone of the epithelium covering the ganglion. Neurites of these randomly distributed cells project directly to the ganglion. The ganglion is very rich in intrinsic FMRFa-ir nerve cells and fibers.

LEnk-ir elements are concentrated in the lateral edges of the lamellae, *i.e.*, in the glandular zone that forms a loop approaching the basal part of the connective-tissue sheath of the ganglion. The LEnk-ir cell bodies are situated just beneath the outer cellular layer of the glandular zone (Fig. 6B, C). Their processes run in two directions. Some of them form a plexus underneath the cell bodies and approach the ganglion running inside the tube-like glandular zone itself. Others run to the ganglion directly, through the ciliated and sensory zones (Fig. 6C). At the periphery of the osphradial ganglion, LEnk-ir fibers form a loose meshwork. Further, there are a small number of LEnk-ir neurons within the ganglion.

MEnk-ir elements of this species are found in the glandular zone of the lamellae (Fig. 6D), as well as in the osphradial ganglion and its surroundings, and are similar to those reacting to antibodies against LEnk. Outside the osphradium, LEnk- and MEnk-ir nerve elements have different distribution patterns.

NADPHd-positive elements are found in the osphradium and concentrated in the ciliated zone of the lamellae, where apical portions of the epithelial cells are stained (Fig. 7A). There are also large, uniform-looking NADPHd-positive cells within the mantle epithelium in the vicinity of the osphradial lamella (Fig. 7B). A few muscle fibers of this particular region also show a positive reaction. No staining was detected after replacement of β-NADPH with α-NADPH.

Non-osphradial epithelia of the gill area

In all species examined, single FMRFa- and LEnk-ir neuroepithelial cells could be seen in the gill and mantle epithelium. Their number is markedly higher in the gill of the pelecypod *Anodonta cygnea* (Fig. 8A, B) than in those of the prosobranchs (Fig. 8C). LEnk-ir cells of these epithelia have a characteristic feature; a strongly immunoreactive layer in the apical part of the cell (Fig. 8B, C). They share this feature with LEnk-ir elements of the osphradial canal in the pulmonate *Lymnaea stagnalis* (Nezlin *et al.*, 1994).

Discussion

Our study has shown that transmitter-specific sensory cells are found in the osphradia of pulmonate and prosobranch molluscs. The FMRFa- and LEnk-ir sensory cells of the osphradium of *Lymnaea stagnalis* (Nezlin *et al.*, 1994) appear also in osphradia of various prosobranch species with different degree of complexity. Thus this work lends support to the idea that transmitter specificity can

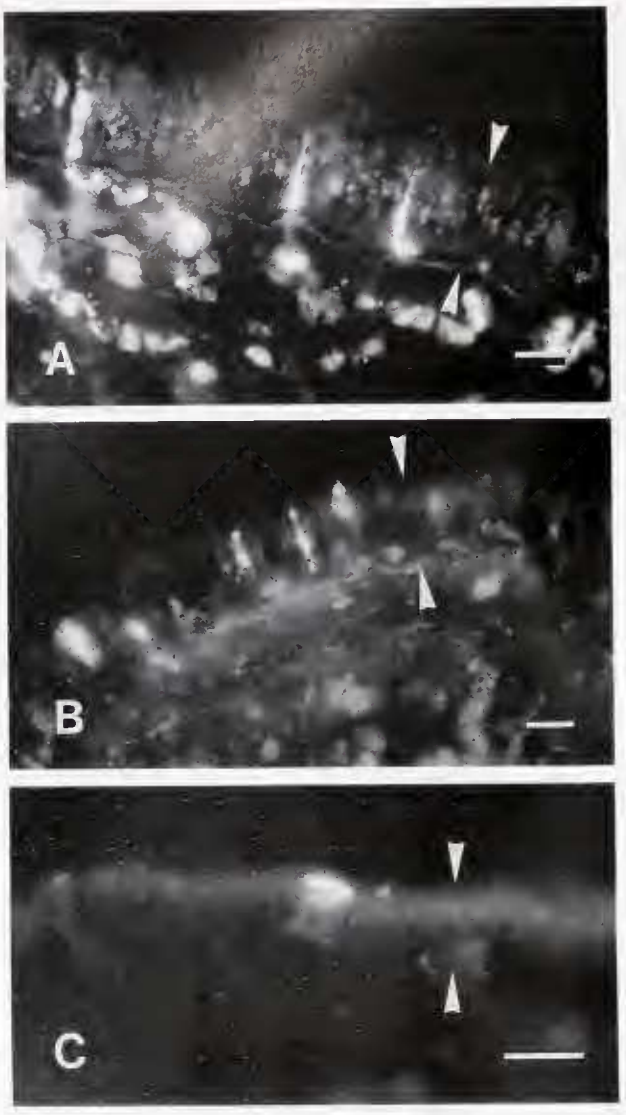

Figure 8. FMRFa-ir (A) and LEnk-ir (B) cells in the gill epithelium of *Anodonta cygnea*, and LEnk-ir (C) cell in the mantle epithelium of *Buccinum undatum*. FITC-labeling. Arrowheads label the borders of the epithelium. Scale bars = 20 μm.

be a conservative feature of homologous nerve cells (Sakharov, 1970).

In the simple osphradium of *Littorina*, all primary sensory neurons labeled with antisera to the two neuropeptides are neuroepithelial cells; that is, they are situated within the epithelium of the organ. This situation refers to the FMRFa-ir sensory cells in the osphradium of *Ampullarius* and *Buccinum*, and to some degree to the LEnk-ir sensory cells. In pulmonates, which are more recent gastropod molluscs, homologous primary sensory cells are true ganglionic neurons incorporated into the osphradial ganglion (Nezlin *et al.*, 1994). Similarly, two ultrastructurally different types of primary sensory neurons were demonstrated underneath the osphradial epithelium in the opisthobranch *Aplysia californica* (Theler *et al.*, 1987). The concentration of specific nerve elements in specific sites and the migration of neuroepithelial cells into central nervous structures are both widely recognized general trends of neural evolution. The sensory cells of the molluscan osphradia seemingly fit well into this model. But there are exceptions like the patellid limpets, which are considered primitive prosobranchs. The cell bodies of their osphradial sensory neurons are situated underneath the epithelium, as in the pulmonates (Haszprunar, 1985). Comparative studies of osphradia might help to elucidate the factors that govern the behavior of neuroepithelial cells, which sometimes migrate from their sites of origin and sometimes, on the contrary, firmly retain their epithelial position.

Reports reviewed by Haszprunar (1985) indicate that, at least in primitive prosobranchs, osphradia are activated by chemical signals from a potential sexual partner. Moreover, sexual arousal of gastropod molluscs is supposedly accompanied by an opioid-dependent suppression of noxious responsiveness and defense behavior (Leonard *et al.*, 1991). An enkephalin-like transmitter released from activated osphradial sensory elements might mediate chemical signals and integrate respective behavioral state.

As for FMRFa-ir sensory elements of the osphradium, they could contain a number of neuropeptides related to, and reactive with, antisera against FMRFamide (Greenberg and Price, 1992; Walker, 1992). At the same time, FMRFamide itself was shown to antagonize the behavioral effects of opioids in molluscs (Kavaliers *et al.*, 1985). We suggest that the two systems of peptidergic sensory neurons of the gastropod osphradium mediate signals inducing opposite behaviors.

Previous examination of the osphradial epithelium by electron microscopy revealed neuroepithelial sensory cells, not only in the central "sensory" zone of the earlier light microscopical investigations, but in the ciliated zone as well (Welsh and Storch, 1969; Crisp, 1973; Haszprunar, 1985). Our results, based on visualization of transmitter-specific elements and summarized in Figure 9, confirm previous conclusions that sensory cells may appear outside of the so-called sensory zone. They also allow us to suggest an extension of the sensory area to zones that are even more lateral to the relatively simple osphradium of *Littorina*. This might lead to a reconsideration of the boundaries of the osphradium to the surrounding mantle wall.

The results show that single FMRFa- and LEnk-ir epithelial cells similar to those occurring in the osphradium can be found in the epithelia covering other organs of the pallial (mantle) complex in prosobranch molluscs. In the

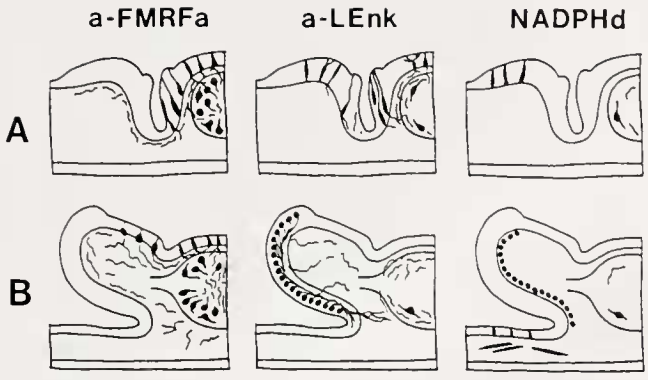

Figure 9. Schematic representation of FMRFa- and LEnk-immunoreactive and NADPHd-positive elements in the left half of the osphradia of *Littorina littorea* (A) and *Buccinum undatum* (B).

bivalve mollusc *Anodonta*, LEnk- and FMRFa-ir cells were found abundantly distributed in the gill epithelium. Correspondingly, bivalves have been described as having poorly differentiated osphradial sensory system (Sokolov and Zaitseva, 1982; Haszprunar, 1987). These findings lead us to speculate that the osphradium may have originated as a site of concentration of epithelial sensory elements that predated the organ itself. The degree of specialization might then have increased progressively during evolution. Note in this connection that, although no LEnk-ir cells were found in or below the epithelium, including the mantle epithelium, of pulmonate molluscs (Sakharov *et al.*, 1993), the concentration of LEnk-ir elements in their osphradial epithelium is extremely high (Nezlin *et al.*, 1994).

MEnk-ir cells were found in a similar position as the LEnk-ir cells only in the highly evolved osphradium of *Buccinum*. In all other prosobranch species, MEnk-ir cell bodies and fibers were distributed in a uniform manner within and around the ganglion. The sensory nature of these cells has not been established, but the involvement of additional sensory subsets of neurons in more sophisticated osphradia is suggested by this finding.

NO attracts wide attention as a novel signal molecule. A possible role for this intercellular messenger in olfactory signal processing has been recently discussed by Breer (1993) and Breer and Shepherd (1993). The osphradium of the pulmonate snail *Lymnaea stagnalis* appears to have been the first invertebrate structure wherein NADPHd-positive cells were demonstrated by a histochemical method and synthesis of NO was proved biochemically (Elofsson *et al.*, 1993). The extent to which this subpopulation of NADPHd-positive elements (the putative NO-producing elements) is present in prosobranch osphradia, and its possible involvement in invertebrate sensory systems, is part of our scientific program. Thus the finding that histochemically positive cells occur in the osphradial region and epithelium was included in this report.

Our results confirm that the dependence of β-NADPH is stereospecific. It was earlier shown by Hope and Vincent (1989) that β-NADPH and α-NADPH gave different staining patterns in the rat brain. Specifically, the alpha form did not stain brain blood vessels that are known to contain NADPH diaphorase related to the NO-synthase (Iadecola, 1993). In our material, no staining at all was obtained with α-NADPH in the osphradia of the two marine species examined, and seemingly nonspecific diffuse staining only was observed in that of *Ampullarius* sp. when α-NADPH was used.

With β-NADPH as a substrate, the NADPHd-positive cells were found in an area next to the osphradium proper in *Littorina* and *Buccinum* and, in addition, seem to be a part of the osphradial lamellae themselves of *Buccinum*. These findings are especially interesting in the case of ciliated NADPHd-positive cells of *Buccinum* in the context of recent results obtained on rat olfactory cilia. A selective inhibitor of NO formation has been shown to prevent completely an increase in cGMP production in response to high odor doses, indicating that, in the rat, NO generated in stimulated receptor cilia may activate guanylate cyclase in adjacent cells (Breer *et al.*, 1992). The NADPH-positive ciliated cells of the gastropod osphradium may provide an advantageous preparation for studying the underlying mechanisms.

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